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Complementary carrier peptide synthesis: General strategy and implications for prebiotic origin of peptide synthesis

(oligonucleotides/templates/catalysis/peptidyl esters)

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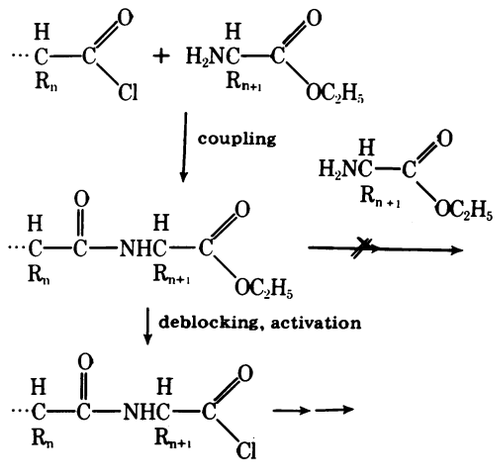
ABSTRACT **A method for peptide synthesis is proposed based on a template-directed scheme that parallels that of the native ribosomal mechanism. In this procedure, peptide bond formation is facilitated by the juxtaposition of aminoacyl and peptidyl oligonucleotide carriers bound adjacent to one another on an oligonucleotide template. The general strategy of the synthesis and relevant model studies are described. The scheme provides an intrinsic mechanism by which oligonucleotides can direct the synthesis of polypeptides in the absence of protein or ribosomal machinery and, as such, suggests a model for the origin of prebiotic protein synthesis.**

With current chemical methods of peptide synthesis, fidelity of the coupling reaction is achieved by the use of a temporary protecting group on either the amine or carboxyl function of the incoming amino acid. For example, for a synthesis from the NH_2 terminus, an ester might serve in this role:



Clearly, it would be desirable to have a method for synthesis that does not require temporary protection in order to achieve fidelity of coupling. In the design of such a synthetic procedure, it has seemed prudent to use as a guide the highly efficient biological mechanism for protein synthesis, which has no requirement for protecting groups whatsoever. This approach has led us to propose a synthetic procedure for peptide synthesis: the complementary carrier method.

Principle of the procedure

Consider (Fig. 1, *upper left*) an oligonucleotide (X) to which the growing polypeptide chain is attached at the 5′-OH by ester linkage to the COOH-terminal carboxyl group and a second, distinct oligonucleotide (Y) to which the incoming amino acid is linked as an ester to the 3′-OH. Although we use here an ester linkage to the carrier oligonucleotides, in principle any substitution-labile acyl linkage might suffice. Anticipating purification steps, we have assumed the NH_2-terminal amino group to be attached to a solid support.

In Eq. 1 the carrier oligonucleotides are brought together for reaction in the presence of the template strand (Z). This strand is composed of two regions, one that is complementary (in the Watson–Crick base-pairing sense) to X and a second that is complementary to Y. There are no intervening nucleotides between the two regions. Eq. 1 represents the preequilibrium binding of X and Y to Z. In the bound configuration, the α-amino group of the incoming amino acid is extremely well placed to attack the peptidyl ester at the 5′-hydroxyl group of X, as can be seen in molecular models (Fig. 2). Eq. 2 represents this reaction. With current chemical methods of peptide synthesis, the ester linkage of the amino acid to Y would have to be substitution inert (blocked) to avoid the undesirable coupling shown in Eq. 3. However, this reaction cannot occur by the template-directed route, but only as a bimolecular process which, by suitable dilution of reactant concentrations, can be made very much slower than the desired coupling of Eq. 2.* Hence, it is feasible to use the substitution-labile (unblocked) linkage between the incoming amino acid and the 3′-OH of Y.

To proceed to the next coupling step of the synthesis we need only wash the unbound oligonucleotides from the resin under conditions that disrupt base pairing; for example, increased temperature or decreased ionic strength (Eq. 4).† In Eq. 5, the next amino acid of the sequence, attached to the 5′-hydroxyl group of X along with the template strand Z, is brought into juxtaposition with the growing peptide chain now attached to Y. In the bound state the α-amino group is well positioned to attack the peptidyl ester at the 3′-OH of Y (Eq. 6). As a result of this reaction, the polypeptide is transferred back to the 5′-hydroxyl group of X. Again, the reaction shown in Eq. 7 can be neglected since this must proceed as a bimolecular process.

The circular sequence of reactions in Fig. 1 can be repeated until the desired polypeptide is constructed. At each step, the growing polypeptide chain is transferred to the complementary carrier oligonucleotide. The polypeptide chain attached to the final carrier would then be cleaved from the solid support. The attached oligonucleotide could serve as a chromatographic handle during the purification of the polypeptide. Finally, the polypeptide would be cleaved under mild alkaline conditions from the terminal carrier.

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Abbreviation: *t*-Boc, *t*-butyloxycarbonyl.

* This follows from the fact that under the conditions of synthesis the desired coupling reaction (Eq. 2) occurs as a first-order process with some fixed effective local concentration of amine (≈1–10 M), whereas the bimolecular side reaction (Eq. 3) is first order in the free concentration of the aminoacyl oligonucleotide ($<10^{-3}$ M).

† In most instances it should prove economically advantageous to recover these oligonucleotides.

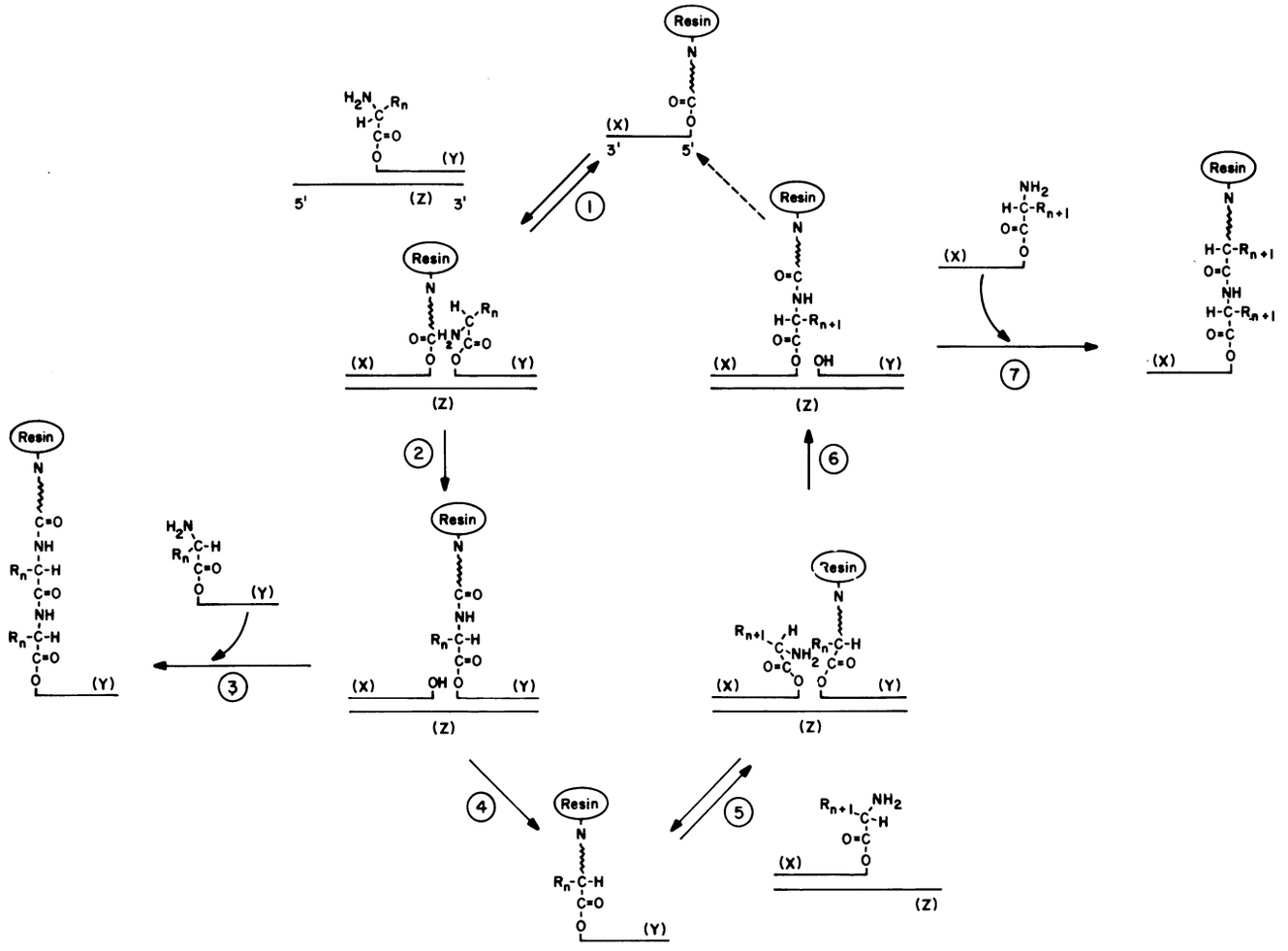


FIG. 1. Complementary carrier scheme for peptide synthesis.

In the present method, fidelity of the coupling reaction is achieved by specific, template-directed catalysis of the desired acyl transfer. Side reactions, although they may occur, do so by a much less favorable bimolecular pathway. This is an essential feature by which fidelity of translation is achieved in the biological mechanism of protein synthesis.

This complementary carrier method offers further advantages. Since a relatively stable acyl linkage to the carriers can be used, due to the fact that acyl transfer is catalyzed by template binding, the synthesis would be carried out in water. This should facilitate proper folding of the peptide chain. In addition, side-chain blocking of the amino acids will be unnecessary in most circumstances since the acyl linkage is stable and orientation for the attack on the peptidyl ester is overwhelmingly favorable to the α-amino group. In practice, only the cysteine SH group and perhaps the ϵ-amino group of lysine would be expected to require protection.

There is already published precedent for template-directed enhancement of reactions between nucleotides and nucleosides similar to that proposed in Fig. 1 (1–3). Most noteworthy are the observations of Chung *et al.* (3) of catalysis of acetyl transfer from 3′-*O*-acetyladenosine to the 5′-OH of adenosine and from 5′-*O*-acetyladenosine to the 3′-OH of adenosine on a polyribouridine template. In these reactions transfer of an acetyl moiety to a well-placed OH group occurs. The chemical transfer that we require (an *N*-acyl amino acid to an amine) should be far more facile than these reactions for three reasons: (*i*) an *N*-acyl amino acid ester is more susceptible to attack than an acetyl ester due to the electron withdrawal by the α-amide substituent; (*ii*) an amine is a much more potent nucleophile than an hydroxyl group; and (*iii*) the scheme uses oligonucleotide carriers, which have far greater affinity for their complementary template than does adenosine alone.

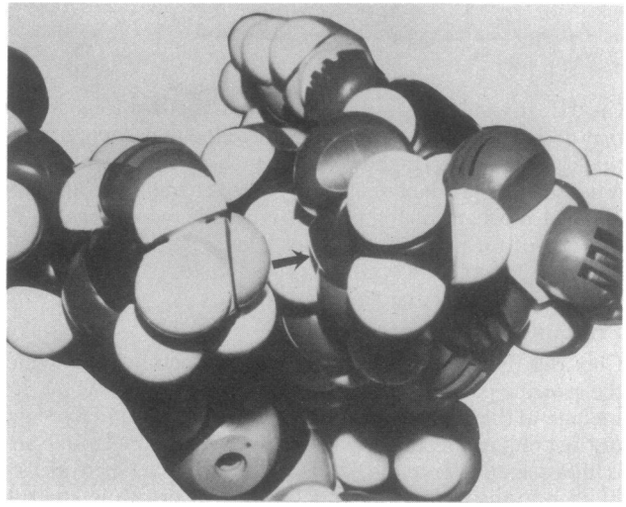


FIG. 2. CPK model of 3′-*O*-glycyl-pTpT bound adjacent to 5′-*O*-acetyl-TpT on the template strand pApApApA. The acetyl group is representative of the growing polypeptide chain. Arrow points from the amino group of glycine to the acyl carbon of the acetyl moiety. The model shows the very close proximity of the attacking amino group to the acyl carbon. No steric hindrance by the attached groups to the formation of the helix is evident.

The scheme lends itself to generalization in two ways. Alternative carriers for polypeptide synthesis can be readily envisioned, although none, perhaps, as practically applicable as are oligonucleotides. We can also anticipate that the complementary carrier method will be extended to the synthesis of other ordered sequence polymers such as polynucleotides.

Materials and methods

3′-*O*-Acetylthymidine and *N*-*t*-Boc-glycine (*t*-Boc, *t*-butyloxycarbonyl) were obtained from Sigma. *N*-Methylimidazole and *N,N′*-dicyclohexylcarbodiimide were purchased from Aldrich. *N*-*t*-Boc-glycine *p*-nitrophenyl ester came from Cyclo Chemical.

5′-*O*-*N*-*t*-Boc-glycyl-3′-*O*-acetylthymidine (I, Eq. 8) was prepared by two procedures. (*i*) *N*-*t*-Boc-glycine anhydride (1 equivalent) was generated from *N*-*t*-Boc-glycine with *N,N′*-dicyclohexylcarbodiimide in pyridine. To this was added 3′-*O*-acetylthymidine (1 equivalent), and the reaction was allowed to proceed at room temperature for several hours. Pyridine was evaporated at reduced pressure. Ethyl acetate was then added and the precipitated dicyclohexyl urea was removed by filtration. The ethyl acetate solution was extracted with water and dried with Na_2SO_4. The product was finally isolated by silica gel chromatography with ethyl acetate as eluant. The R_F value is 0.47 with ethyl acetate on silica thin-layer plates (Kontes, K6F). The spot was ninhydrin positive after deprotection with HCl fumes. Analysis of product: calculated for $C_{19}H_{27}N_3O_9$; C, 51.69, H, 6.17, N, 9.52; found; C, 51.56, H, 6.15, N, 9.37. (*ii*) To a solution of *N*-*t*-Boc-glycine *p*-nitrophenyl ester (1.1 equivalents) and 3′-*O*-acetylthymidine (1 equivalent) in pyridine was added 10 equivalents of *N*-methylimidazole, and the mixture was allowed to stand overnight at room temperature. The product was then isolated as in procedure *i*. It was identical to that from procedure *i*.

The reaction between 5′-*O*-*N*-*t*-Boc-glycyl-3′-*O*-acetylthymidine and glycinamide (Eq. 8) was followed by analytical high-pressure reverse-phase chromatography with a Perkin–Elmer series 2 liquid chromatograph. Pseudo-first-order rate constants for the conversion of 5′-*O*-*N*-*t*-Boc-glycyl-3′-*O*-acetylthymidine to 3′-*O*-acetylthymidine, k_{obs}, were fitted by least-squares analysis to the equation

$$k_{obs} = k_0 + k_n[\text{glycinamide}] + k_{a,b}[\text{glycinamide}]^2,$$

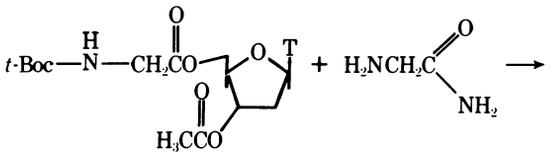
in which k_0 corresponds to the spontaneous rate of hydrolysis, k_n to the rate for nucleophilic attack by glycinamide, and $k_{a,b}$ to the sum of the rates for general acid and general base catalysis by glycinamide of the attack of glycinamide on the *N*-*t*-Boc-glycine ester. At 22°C, pH 7.5, and ionic strength 1.5 M (glycinamide varied from 0 to 1.0 M), these rate constants were 1.4×10^{-4} min^{-1}, 2.3×10^{-4} min^{-1} M^{-1}, and 2.9×10^{-4} min^{-1} M^{-2}, respectively.

Specifications for oligonucleotides as complementary carriers

Suitable carriers must first have the requisite complementary binding properties, as clearly oligonucleotides do. From a variety of studies on helix stability it appears unlikely that carriers greater than eight nucleotides in length will be necessary (1, 4–7). Of course, the precise length needed will depend on the base sequence and on the conditions, particularly the temperature, under which the coupling reactions are carried out. Carrier oligonucleotides of this approximate length, as well as the corresponding template strand, are well within the range of current methods of oligonucleotide synthesis (8–11).

The linkage of the amino acids to the carrier (the actual chemical bond as well as the stereochemistry) must satisfy the following criteria: (*i*) thermodynamically, peptidyl transfer (Eq. 2) must proceed far in favor of peptide bond formation; (*ii*) back transfer of the peptide chain to the site of attachment on the previous carrier must not take place (e.g., there must be no transfer of the polypeptide from the 3′-OH of Y back to the 5′-OH of X after the coupling reaction in Eq. 2); and (*iii*) the rate of peptide bond formation (aminolysis) must be greater than 5000 times that for hydrolysis of the peptide from the carrier. The specific value of 5000 arises from the following argument. For each coupling we must allow 15 half-lives of the aminolysis reaction to assure sufficiently high yield (>99.99%) of peptide transfer. For a protein 300 amino acids in length, this represents a total of 4500 half-lives. Clearly, if the half-life for hydrolysis of the peptide from the carrier (and hence, chain termination) were much less than this value, the yield of the synthesis would fall unacceptably low. It is relevant to point out that chain termination due to hydrolysis of the peptide from the carrier does not present a purification problem because the cleaved chains will be distinguishable from those that are attached at the COOH terminus to the final carrier oligonucleotide, a convenient affinity handle for chromatographic isolation.

The most obvious choice for the attachment of the amino acids to the oligonucleotide carriers is through oxygen ester linkage as shown in Fig. 1. To examine the suitability of this mode of attachment we studied the model reaction:



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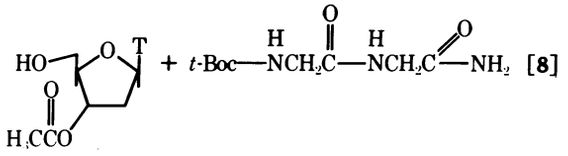
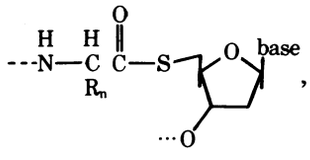


[8]

The second-order rate constant for this reaction is 2.3×10^{-4} M^{-1} min^{-1} at 22°C and pH 7.5. Under the same conditions, the pseudo-first-order rate constant for the hydrolysis of the *N*-*t*-Boc-glycine ester is 1.4×10^{-4} min^{-1}. Thus, if the rate for the intramolecular aminolysis reaction (Eq. 2) is to be 5000-fold the pseudo-first-order rate of hydrolysis, the effective intramolecular concentration of the amine would have to be approximately 3000 M. This value is larger than can be expected on the basis of proximity effects alone and indicates the need for specific catalytic enhancement, as is provided by peptidyltransferase in the native ribosomal mechanism. Alternatively, we can take advantage of the fact that aminolysis of esters is very sensitive to the nature of the leaving group whereas hydrolysis is not (12). In particular, attachment of the amino acids to the oligonucleotides by a thiol ester linkage instead of an oxygen ester,



would be expected to greatly enhance aminolysis rates (i.e., peptide transfer) but to change the rate of hydrolysis little if at all (12, 13). Variations in the mode of attachment of the amino

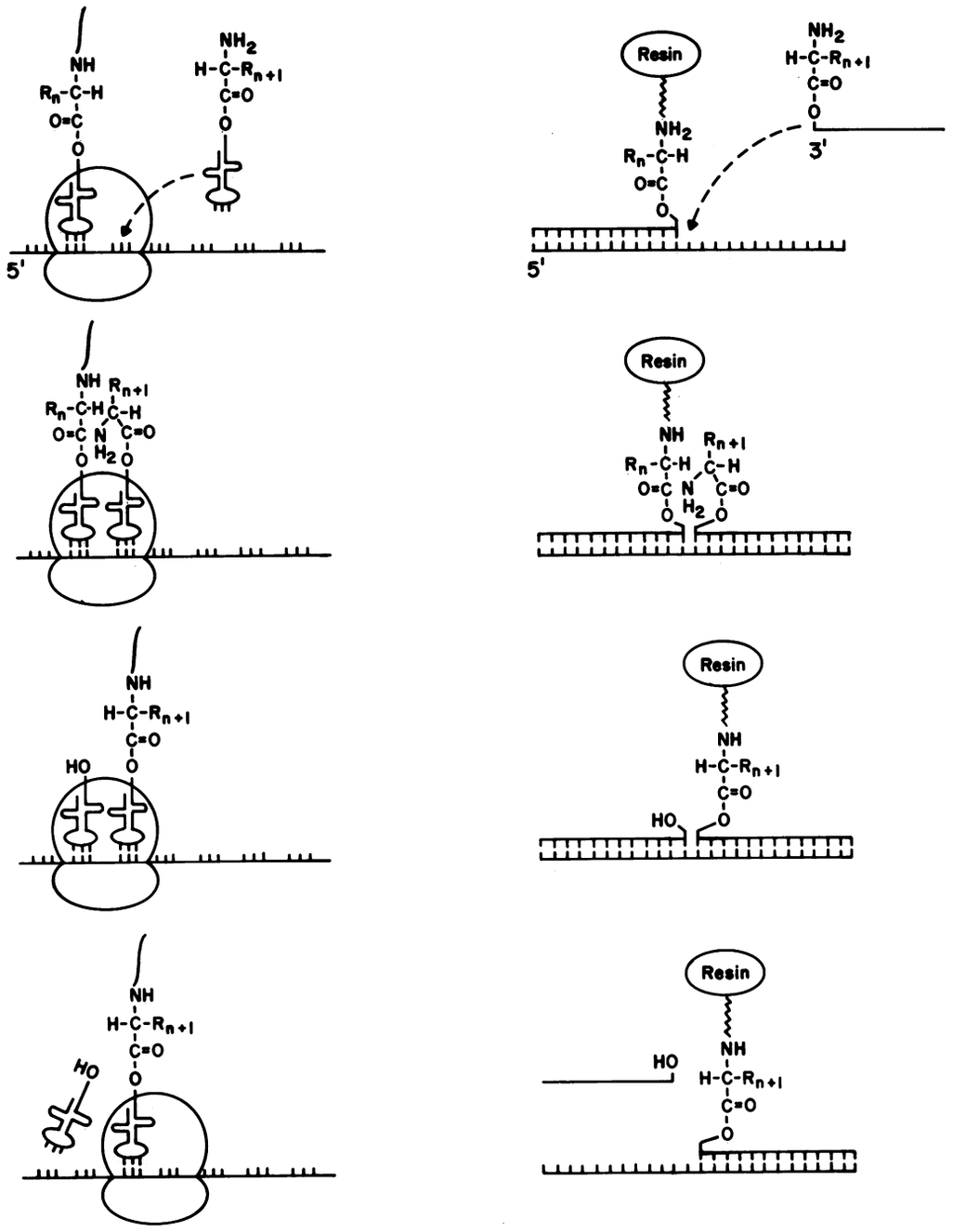


FIG. 3. Comparison of the complementary carrier scheme for peptide synthesis and the natural ribosomal mechanism.

acids to the oligonucleotide carriers represent an important point of flexibility in our synthetic scheme.

Implications for prebiotic evolution of protein synthesis

There are two important biological functions of nucleic acids: replication and the direction of protein synthesis. The latter fundamentally provides a means of self protection for the nucleic acid molecule. It seems most probable that present-day systems for carrying out these functions evolved from mechanisms, although much less efficient, inherent to polynucleotides. Watson–Crick base pairing provides the intrinsic mechanism for achieving fidelity of replication. We suggest that the catalysis of peptide bond formation through the juxtaposition of peptidyl and aminoacyl oligonucleotides on a polynucleotide template, as in Fig. 1, provides the intrinsic mechanism for the direction of protein synthesis. Initially there would have occurred only the synthesis of random-sequence polypeptides, the results of acyl transfer between the 3′ and 5′ ends of oligonucleotides. In time, the carrier oligonucleotides evolved into more efficient species (tRNA) which, when bound on a polynucleotide template, folded so as to approximate their 3′ ends.‡ The appearance of tRNA capable of 3′ → 3′ acyl transfer set the stage for the development of the genetic code and the accumulation of a linear array of information. With further evolution, the biosynthesis of proteins attained the form in which it exists today. Nevertheless, the underlying mechanism of acyl transfer between a peptidyl oligonucleotide and an aminoacyl

‡ Whatever the mechanism postulated as the initial form of protein synthesis, only random-sequence polypeptides could have been produced at first since there was no genetic information to transcribe. Most likely, selective pressure would have been exerted to increase the quantity of protein synthesis. In this way species of RNA capable of some cooperative interaction and thereby 3′ → 3′ acyl transfer may have offered the advantage of requiring shorter stretches of complementarity to bind to template.

oligonucleotide bound adjacent to one another on a polynucleotide template has been preserved (see comparison in Fig. 3).

It has been suggested (14) that protein synthesis was initially carried out by oligonucleotides alone, without the assistance of protein or ribosomal machinery. All previously described mechanisms, however, have been based on 3′ → 3′ acyl transfer and some recognition of amino acid by the oligonucleotide carrier. Although we believe this represents an obligatory stage through which the evolution of protein synthesis must have passed, it corresponds to the accumulation of a large amount of sequence information and seems unlikely to have been the origin of protein synthesis. The original synthetic mechanism must have depended solely on properties inherent to polynucleotides, such as in the model proposed in Fig. 1. Studies of our synthetic procedure should allow us to define more precisely the efficiency of this intrinsic pathway of polypeptide synthesis.

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